

STUDIES ON DOPA METABOLISM IN TWO
DIFFERENT KINDS OF TUMOUR

BY

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LUND 1972



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OF TUMOUR

By

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This thesis is based on the following publications:

- I. On the occurrence of catechol derivatives in malignant melanomas. Communications from the Department of Anatomy, University of Lund, Sweden No 5, 1-8, 1966 (Together with B.Falck, S.Jacobsson, H.Olivecrona, H.Rorsman and E.Rosengren)
- II. Fluorometry of a dopa peptide and its thioether. Acta Dermatovener (Stockholm). 51, 179-182, 1971 (Together with H.Rorsman and E.Rosengren)
- III. Fluorometry of catechol thioethers. Acta Dermatovener (Stockholm) - In the Press (Together with H.Rorsman and E.Rosengren)
- IV. Fluorometry of a dopa peptide and dopa thioethers. Proc. 8th. Pigment Cell Conference, Sidney, 1972. Karger, In the Press (Together with H.Rorsman and E.Rosengren)
- V. Catechol derivatives in the skin of young mice. Acta Univ. Lund. II, No 30, 1-5, 1967 (Together with L.Cegrell and B.Falck)
- VI. Extraction of dopa from the integument of pigmented animals. Acta physiol. scand. 78, 65-69, 1970 (Together with L.Cegrell and B.Falck)
- VII. Dopamine and 5-hydroxytryptamine in the guinea-pig pancreas. Life Sci. 6, 2483-2489, 1967 (Together with L.Cegrell and B.Falck)
- VIII. Catechol and indole derivatives in a transplantable islet-cell tumour of the golden hamster. Acta physiol. scand. 77, 23-27, 1969 (Together with L.Cegrell and B.Falck)
- IX. Dopamine binding in a transplantable islet cell tumour of golden hamster. Acta path. microbiol. scand. Section A. 79, 463-466, 1971 (Together with L.Cegrell, L.Nordgren and E.Rosengren)
- X. Tyrosine hydroxylase activity in a transplantable islet cell tumour of golden hamster. Experientia 26, 998-999, 1970 (Together with S.Axelsson and L.Cegrell)
- XI. Dopa-decarboxylase and monoamine oxidase activities in a transplantable islet cell tumour of the golden hamster. Experientia 25, 969-970, 1969 (Together with L.Cegrell and B.Falck)

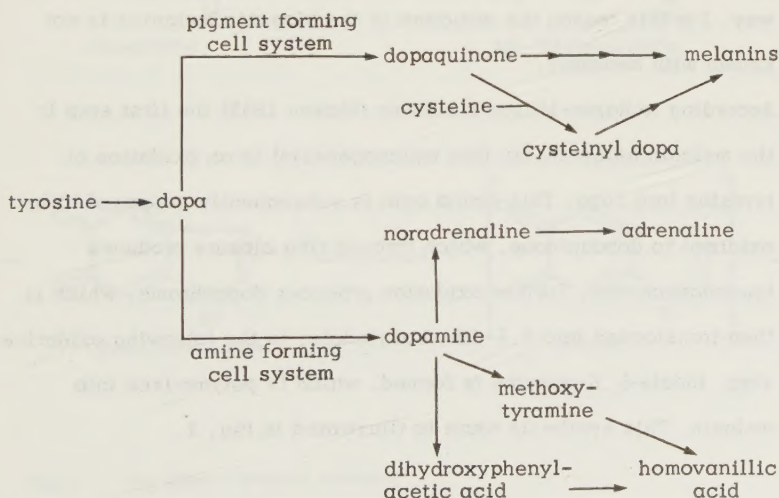
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INTRODUCTION

Dihydroxyphenylalanine (dopa) is not an essential amino acid, since it is formed in the body from tyrosine. Its biological importance lies mainly in the fact that it is a precursor of the catecholamines, the group of substances believed to be transmitters in the central nervous system and recognized as such in the peripheral adrenergic nervous system. Further, dopa is a probable intermediate in the synthesis of pigment.

This thesis discusses the occurrence and metabolism of dopa and compounds derived from this amino acid in two different kinds of cell: the pigment forming cells and the endocrine cells in the pancreas. Emphasis is mainly laid on two tumours, namely melanin producing malignant melanomas, and a dopamine producing insuloma. The pertinent feature of dopa metabolism is illustrated in the following scheme:



DOPA IN PIGMENT FORMING TISSUES

It is generally agreed that the various colours characteristic of the pigmentation of skin, hair, and choroid in mammals are produced mainly by two different classes of melanins: eumelanins, which are mostly black, and phaeomelanins, which are brown, red-brown or red. This classification is based on the assumption that the units included in the irregular polymer constituting the melanins are different. Thus the eumelanins are supposed to derive exclusively from oxidation products of tyrosine, whereas the phaeomelanins are supposed to be formed from tyrosine and cysteine. Most melanins are conjugated with proteins. Studies on sepiomelanin have revealed that in this case the pigment is bound to protein by means of a cysteine unit (Piattelli et al. 1963). In order to separate the melanin from the protein matrix, hydrolysis with strong hydrochloric acid is necessary. It is reasonable to assume that acid hydrolysis causes a destruction of the native melanin molecule in a not fully understood way. For this reason the structure of the biogenic melanins is not known with certainty.

According to Raper-Mason's scheme (Mason 1953) the first step in the melanin biosynthesis (the melanogenesis) is an oxidation of tyrosine into dopa. This amino acid is subsequently supposed to be oxidized to dopaquinone, which through ring closure produces leucodopachrome. Further oxidation produces dopachrome, which is then transformed into 5,6-dihydroxyindole. In the following oxidative step, indole-5,6-quinone is formed, which is polymerized into melanin. This synthesis route is illustrated in Fig. 1.

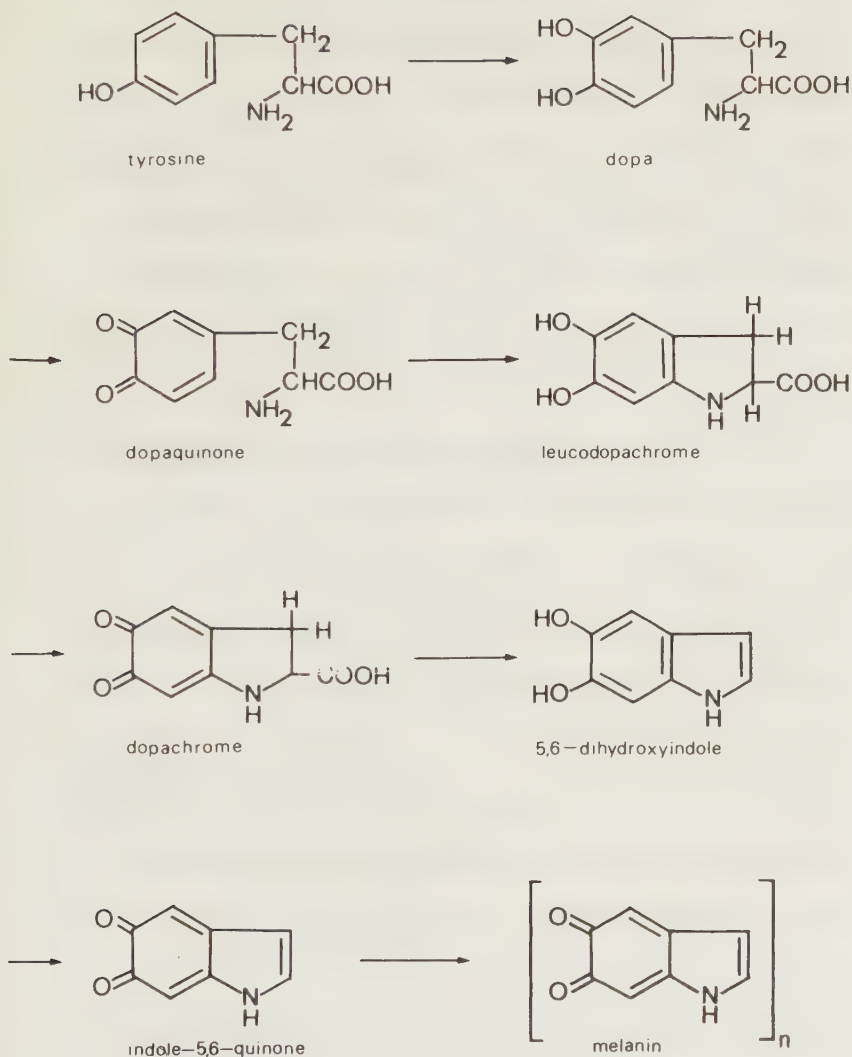


Fig. 1 The Raper—Mason's scheme

It would at present appear that the former concept of eumelanins as constructed solely as a polymer of indole quinones is not altogether correct. Work by, among others, Chen and Chavin (1966) and Hempel (1966), shows that the carboxyl group of the tyrosine is found in the melanin, not only in indole quinone units, but also in dopaquinone units. According to this concept, melanin consists of a macromolecule formed by polymerization of dopaquinone, indole-5,6-quinone and indole-5,6-quinone-2-carboxylic acid. The present work is aimed to show that dopa itself is included in melanin. Fig. 2 shows the hypothetical formula for the structure of melanin proposed by Hempel (1966).

There are few reports on record on phaeomelanins, and these include no detailed analysis of the units that compose the macromolecule. However, a low molecular subgroup of the phaeomelanins, called trichosiderins has recently been described. The name trichosiderins was given in 1945 to a group of red pigments isolated from hair of man by extraction with boiling 0.1 N HCl (Flesch and Rothman 1945). It was earlier considered that these pigments contain iron in the molecule - thus the name - ; yet, this proved later not to be the case. These pigments are not conjugated to protein, a fact that has facilitated their isolation and identification. The formula of a pigment belonging to the group trichosiderins is illustrated in Fig. 3, as a representative of the structure of a phaeomelanin (Fattorusso et al. 1969). This benzothiazine derivate, like other phaeomelanins, is supposed to originate from a reaction between dopaquinone and cysteine (Prota et al. 1968).

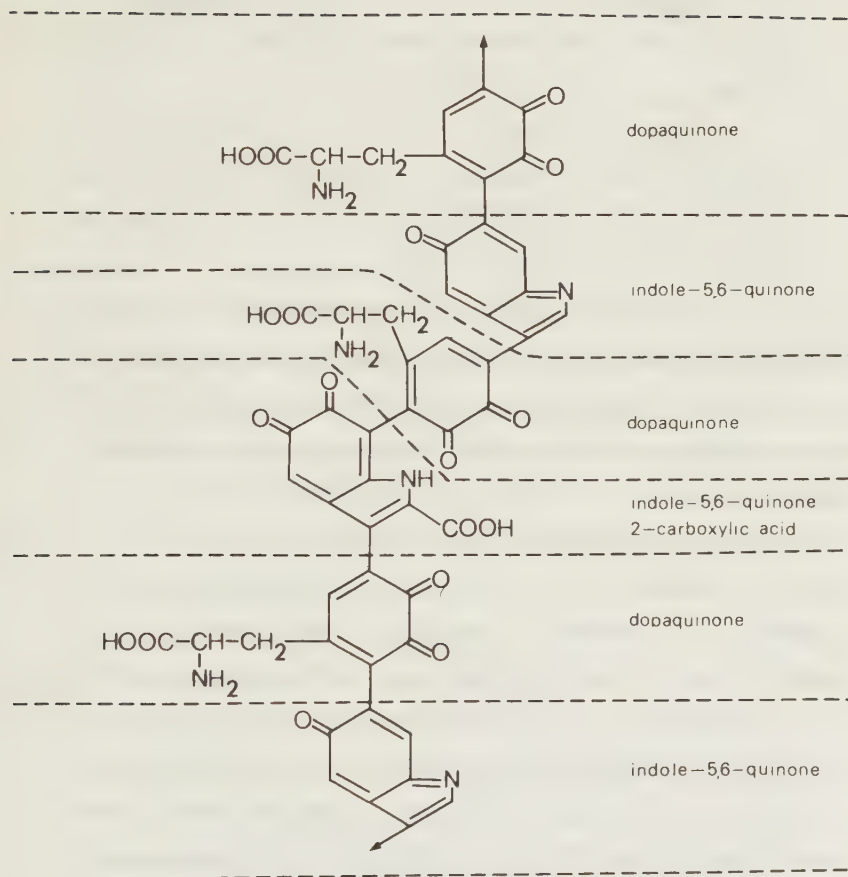


Fig.2 Melanoma melanin according to Hempel

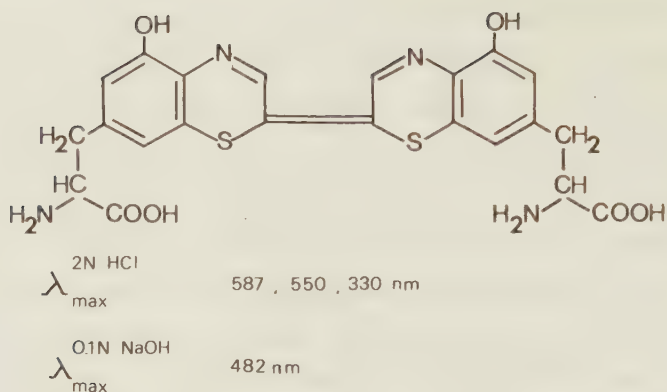


Fig.3 The structure of a trichosiderin, designated F1.

It is now generally assumed that dopa is an intermediate in the biosynthesis of both eumelanin and phaeomelanin. The fact that dopa can easily be converted into melanin favours this view. There are, however, other compounds which in the body can be converted to melanin, such as certain indole derivatives. The issue would be strengthened by demonstrating dopa in the pigment forming cell, which only lately has been possible. The demonstration of dopa in calf eyes was a discovery of significant importance (Bernheimer 1963). This author lacked the possibility of localizing the amino acid at the cellular level, but he supposed that it occurred in pigment forming cells rather than in catecholamine containing nerve fibres. In independent work the occurrence of dopa in some pigment forming tissues has subsequently been reported (see papers I, V and VI, and also further references in these papers).

Catechol compounds in human melanomas

Human malignant melanoma tissue that has been subjected to treatment by the histochemical formaldehyde method developed by Falck and Hillarp (for ref., see Falck and Owman 1965), shows an intense green-yellow to yellow fluorescence in the tumour cells (Falck et al. 1965). This fluorescence, which indicates the presence of certain catechol- or indole compounds (Björklund et al. 1971), was tentatively interpreted as indicating the presence of dopa. In fact, Hempel and Männl (1966), who injected melanoma-carrying mice with radioactive tyrosine, could demonstrate dopa formation in the tumour. Owing to the lack of a sensitive and specific method for the determination of dopa the demonstration of endogenous dopa in melanoma tissue was not possible at that time. Employing an improved method for determining of dopa, based on Anton and Sayre's (1964) Al_2O_3 adsorption method, dopa could be demonstrated in human and also in experimental malignant melanomas in 1966 (paper I). Other reports appearing at the same time (Falck et al. 1966, Pomerantz and Warner 1966, Takahashi and Fitzpatrick 1966) support this observation.

Analysis of human pigmented melanoma tissue (paper I) showed an average dopa content of $1.1 \mu\text{g/g}$. On fluorometric analysis, besides the spectral curves of dopa (330/380), a fluorescence maximum at about 500 nm was revealed. In accord with this analysis, paper chromatography of eluates obtained from melanomas and exposure of the chromatograms to formaldehyde at $+80^\circ\text{C}$ for one hour (the same type of treatment used for tissues by Falck and Hillarp) revealed two spots fluorescing in UV-light. Both spots, one of which showed a R_f -value corresponding to dopa, emitted an intense yellow-green light.

After treatment with $K_3Fe(CN)_6$, both spots showed red colour. It thus appears that human malignant melanomas contain, besides dopa, at least one more catechol derivative.

Hydrolysis with hydrochloric acid of the precipitate obtained at the perchloric acid extraction yielded high amounts of dopa, approximately 20 times more than was found in the supernatant. This striking finding is at present difficult to explain, but unpublished experiments, in which eye melanin was hydrolyzed for a long time under the same conditions, indicate that the additional dopa originates from a splitting of the melanin polymer itself. If this is the case, this would further support the view that dopa is a constituent of melanin, a proposition that remains to be critically confirmed. Alternatively, it could be speculated that dopa is adsorbed to melanin, but this must be discounted, because dopa could not be detected at fluorescence microscopy of the eye melanin (B. Ehinger, personal communication). Another alternative route lies in the possibility that tyrosine formed by hydrolysis could be oxidized to dopa, in the presence of melanin. This is not the case since the addition of large amounts of tyrosine to eye melanin followed by boiling in 6 N HCl produced no increase in the amount of dopa (unpublished results).

The identification of the unknown compound would be easier if the agent occurred in melanomas that are more easily available than the human. In paper I, three different types of mouse melanomas (B 16, Harding-Passay, and S 91) and a hamster melanoma (M Mel 7) were investigated for their contents of catechol derivatives. All specimens contained dopa, in some cases in concentrations of the same magnitude as in human melanomas. Extraction of Harding-Passay melanoma with

a 25 per cent urea solution showed that here dopa was not firmly bound, since this gentle procedure yielded the same amounts of dopa as did extraction with perchloric acid (unpublished observations). Dopamine, determined by spectrophotofluorometry according to Bertler et al. (1958), did not occur in detectable amounts. In mouse melanoma, dopa decarboxylase activity, determined according to Bertler and Rosengren (1959), was found to be low, whereas no such activity could be demonstrated in the hamster melanoma. It is noteworthy in this context that in the hamster melanoma M Mel 1, besides dopa, both dopa decarboxylase activity and high amounts of dopamine have been demonstrated (Håkanson et al. 1965). On paper chromatographic analysis of the experimental melanomas, no evidence of the occurrence of catechol derivatives, besides dopa, was obtained. This and the fact that neither hamster melanoma nor mouse melanoma fluoresce after treatment with formaldehyde according to Falck and Hillarp (Falck et al. 1969) indicate that the formaldehyde induced fluorescence in human malignant melanomas is due to a catechol compound other than dopa. In this respect the experimental melanomas showed definite differences compared with the human melanomas.

Further attempts at identifying the unknown agent was hampered by the great difficulty in obtaining human melanoma tissue in amounts adequate for chemical analysis. Independent of the supply of tumour tissue a hypothetically conceivable possibility that could be tried, namely that the unknown fluorophore was not derived from dopa, but from dopa bound to amino acids, either in peptide- or thio-ether bindings. This question was approached in experiments with various

model substances (papers II and III). In a first series of experiments (paper II), tyrosyl-glycyl-glycine (TGG) was incubated with tyrosinase as described by Yasunobu et al. (1959) and the dopyl-glycyl-glycine (DGG) formed was isolated according to Anton and Sayre (1964). When this short peptide was oxidized with periodate according to Anton and Sayre or exposed to formaldehyde (on paper chromatograms) according to Falck and Hillarp, the spectral characteristics of dopa were obtained. These findings strongly indicated that the unknown catechol derivative in human melanoma is not a dopyl peptide with dopa in N-terminal position. Dopa in C-terminal position should lack the ability to produce fluorescence with formaldehyde and could therefore be excluded.

In another series of experiments, TGG was incubated with tyrosinase and cysteine (paper II). A catechol isolation was performed according to Anton and Sayre (loc. cit.). Spectrophotometry and spectrophotofluorometry revealed the appearance of a catechol derivative that probably was a thio-ether of DGG and cysteine (CDGG). Thus, an absorption spectrum similar to that reported by Bouchilloux and Kodja (1960) for thio-ethers formed by dopaquinone and cysteine or glutathione was obtained. Further, the fluorescence spectra agreed with those characteristic of authentic 5-S-cysteinyl dopa after oxidation in the same way. Moreover, the incorporation of cysteine into the substance formed was proved by addition of ^{14}C or ^{35}S labelled cysteine.

These fluorescence spectra were identical with those obtained with the unidentified catechol present in human melanoma indicating the existence of a compound with sulphur bound to the benzene ring in

dopa, similar to thio-ethers. This result prompted to systematic exploration with model experiments reported in paper III. In the present work, tyrosinase was used for the synthesis of reference substances. Tyrosine, TGG, or tyramine were incubated either with cysteine or with glutathione and the catechols formed were isolated by adsorption on Al_2O_3 . As in the studies of Bouchilloux and Kodja (loc.cit.) the compounds obtained all had absorption maxima at 255 and 292 nm. Subsequent spectrophotofluorometric analysis gave the following results (wave lengths corrected with quinine-sulphate in 0.1 N H_2SO_4);

- 1) Enzymatically produced cysteinyl dopa (Fig. 4) displayed activation/emission maxima at 355/485 nm, i.e. at longer wave lengths than those for dopa (330/380).
- 2) The emission maximum for CDGG was 470 nm, whereas its excitation maximum was the same as for cysteinyl dopa.
- 3) Glutathione dopa and glutathione DGG had excitation maxima at wave lengths laying between the maximum for dopa and the maxima for cysteinyl dopa and CDGG. The emission maxima for the glutathione compounds were almost the same as those of dopa, but there was a slope at a somewhat longer wave length. The fluorometric analysis presented in paper III excludes the possibility that the catechol derivative in human malignant melanomas is a glutathione-catechol compound. Further, glutathione dopa did not turn violet after heating (see below). This study also excludes the possibility that the unknown catechol is cysteinyl dopamine as at oxidation it had no fluorescence at the wave lengths corresponding to those of the fluorophore of the unknown substance.

A study of a melanoma from a patient with red hair is presented in paper IV. This tumour was found to contain large amounts of the unknown catechol derivative which exhibited the following characteristics:

- 1) It had an absorption spectrum with maxima at 255 and 292 nm and a minimum at 275 nm, i.e. identical to those of cysteinyl dopa (Fig. 5).
- 2) On paper electrophoresis and paper chromatography the substance moved as cysteinyl dopa.
- 3) After oxidation with periodate or after treatment with formaldehyde it had the same fluorescence characteristics as cysteinyl dopa.
- 4) The compound turned violet after heating to 100°C in 0.1 N HCl and the product formed had absorption maxima at 330 and 587 nm in 2 N HCl (Fig. 6) and at 482 nm in 0.1 N NaOH (cf. Fig. 3). This reaction is also characteristic of cysteinyl dopa (Prota, personal communication).

These findings strongly support the view that the unknown catechol derivative in human malignant melanomas described in paper I is 5-S-cysteinyl dopa (Fig. 4).

Catechol compounds in normal skin

The direct demonstration of dopa in pigment forming cell systems suggests that dopa is an intermediate in the synthesis of pigment. In paper V the occurrence of catechol derivatives was investigated in skin from both pigmented and albinotic mice, 4-11 days old. In pigmented skin subjected to the histochemical fluorescence procedure, intensely green fluorescent granular cells were observed in dermis and subcutis. Staining with toluidine blue showed that these

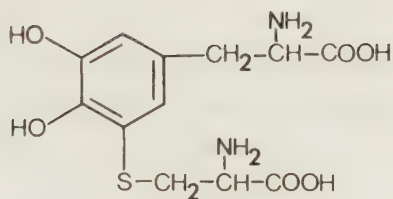


Fig. 4 Cysteinyldopa

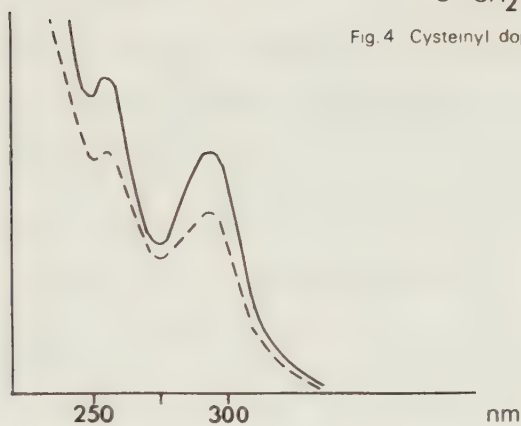


Fig 5 Absorption spectra of material from melanoma (---) and enzymatically produced cysteinyldopa (—).

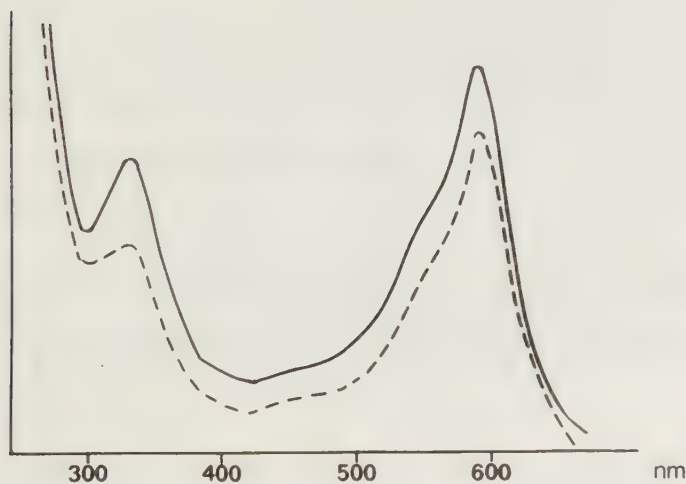


Fig. 6 Absorption spectra of heated material from melanoma (---) and enzymatically produced cysteinyldopa (—)

cells were metachromatic, and thus by definition mast cells. A further cell system which showed a weak green to yellow-green formaldehyde induced fluorescence was found in hair matrix of the pigmented animals. In the skin of albinotic mice, the mast cells showed a very weak fluorescence, and no fluorescing cells could be observed in hair matrix. Chemical determinations showed that about the same amounts ($0.8 \mu\text{g/g}$ or less) of both dopa and dopamine occurred in the pigmented skin. The identity of the substances could be confirmed by paper chromatography. Detectable amounts of catechol derivatives were not found in skin of white mice. At the time of this study no means were known to differentiate dopa and dopamine at the cellular level. A reasonable assumption is that the dopamine found was stored in mast cells. It is known that monoamines are stored in the mast cells in many mammalian species. It has also been demonstrated that mast cells in hamster and rabbit can take up L-dopa, which in the cell is decarboxylated to dopamine (Adams-Ray et al. 1964, 1965). It is a remarkable fact that, at a certain phase of development, the monoamine occurring in mast cells of the mouse is dopamine, whereas in the adult mouse the mast cells contain 5-hydroxytryptamine (Cass et al. 1958). The specific, i.e. the formaldehyde induced fluorescence in some hair matrix cells is believed to derive from dopa, a supposition that is strongly supported by continued investigations (paper VI). In report VI the occurrence of catechol compounds was investigated in hair and shaved skin from the guinea-pig, rabbit, and dog. Experimental details, extraction methods employed, and the results with the chemical determinations are summarized in Table 1.

Table 1. Dopa content in hair and shaved skin in different species. The figures express $M \pm SEM$ or values of single determinations.

Species	Extraction agent	Number of determinations	Dopa $\mu\text{g/g}$ wet weight	
			Hair	Shaved skin
Pigmented guinea-pigs	boiling water	7	2.1 ± 0.44	0.29 ± 0.10
	perchloric acid	7	0.90 ± 0.16	0.10 ± 0.007
Albino guinea-pigs	boiling water	2	0.09 and 0.14	0.03 and 0.03
Multi-coloured guinea-pigs:				
white parts	boiling water	2	0.14 and 0.22	0.00 and 0.02
coloured parts	boiling water	2	3.4 and 5.3	0.27
Pigmented guinea-pigs	ethanol	2	0.55 and 0.97	-
Pigmented young rabbits	boiling water	4	$8.5; 12; 23; 24$	1.9 and 3.8
	perchloric acid	2	2.4 and 5.7	0.76 and 0.86
Pigmented adult rabbits	boiling water	1	1.3	0.05
	perchloric acid	1	0.46	0.02
Pigmented dogs	perchloric acid	3	$4.6; 4.7; 5.6$	0.63 and 1.0

As seen from Table 1, mainly two extraction methods were used: namely perchloric acid and boiling water. The object was a comparison of a mild extraction procedure on one hand and perchloric acid extraction on the other to see whether the dopa could be formed by cleavage of compounds in the tissue. It is noteworthy that extraction with boiling water yields considerably larger amounts of dopa than extraction with perchloric acid or ethanol, whereas perchloric acid proved to be the most effective extraction means for dopa in other tissues (unpublished results). At present, this finding cannot be satisfactorily explained; a circumstance that perhaps played a role is that keratinized tissue was the material investigated. The hair matrix in the pigmented skin of guinea-pigs and rabbits was found to contain melanocytes which displayed a formaldehyde induced green-yellow fluorescence. Similar results have been reported by Olivecrona and Rorsman (1966). Because of an intense autofluorescence, the possible occurrence of a specific fluorescence could not be evaluated in hair. As shown in Table 1 the dopa content of unpigmented tissue was low also in white skin areas from multi-coloured guinea-pigs. This finding, and the fact that a specific fluorescence could only be observed in melanocytes in hair matrix, indicate that in the skin dopa is correlated to pigment formation. The specific fluorescence of cells in guinea-pig skin showed, in microspectrofluorometric measurements, an excitation maximum at 430 nm, whereas emission maximum varied between 480 and 520 nm. This variation was tentatively interpreted as due to the occurrence of more than one fluorophore in different proportions. This hypothesis has gained increased current interest after it was shown that in tissues

derivatives of dopa can occur which with formaldehyde can produce a fluorophore whose spectral characteristics deviate from those of the dopa fluorophore (paper IV). The occurrence of dopa in hair is a surprising finding. This localization can perhaps be explained by the accumulation of dopa in hair in connexion with transport of pigment.

DOPA AND DOPAMINE IN PANCREAS AND INSULOMA

In neural tissue, dopa is metabolized entirely differently than in pigment forming cell systems. The metabolic steps, proposed by Blaschko in 1939 are as follows:



At that time, the importance of adrenaline as a hormone of the suprarenal gland was known. Noradrenaline was shown by von Euler (1946) to be present in the peripheral sympathetic nervous system in mammals, and Holtz (1950) demonstrated the occurrence of noradrenaline in the brain. Considerable amounts of dopamine were demonstrated in certain peripheral organs: in heart of the sheep (Godall 1951), in the sympathetic nerves of the cow (Schümann 1956), in lung and spleen of the cow (Euler and Lishajko 1957). Bertler and Rosengren (1959 a) found dopamine in these peripheral organs only in ruminants, and in such tissues the amine seemed localized exclusively to mast cells (Falck et al. 1964). The presence of dopamine also in other organs has been demonstrated (for ref. see paper VII).

The functional significance of dopamine in mast cells and other peripheral cell systems is unknown. Montagu, in 1957, reported the finding of dopamine in the brain, confirmed by Carlsson et al. (1958). Bertler and Rosengren (1959) showed that dopamine in brain of mammals is mainly localized to the caudate nucleus and the putamen, i.e. to areas in the brain that contain very small amounts of noradrenaline. These findings led to the assumption that dopamine has a function of its own in the brain and not merely exists there as precursor to noradrenaline.

Dopamine in endocrine pancreas

A specific monoamine fluorescence was demonstrated in endocrine pancreas in a number of different mammals (for ref., see Cegrell 1968 and paper VII). Report VII presents a chemical and micro-spectrographic study of the occurrence of monoamines in guinea-pig pancreas. The monoamine determinations were carried out with the spectrophotofluorometric method of Bertler et al. (1958) and were supplemented by paper chromatographic analysis of the catecholamines in three different systems. The cellular localization of the monoamines was carried out employing the histochemical fluorescence method. The experimental material consisted of 50 to 65-day old fetuses, newborn, and adult animals.

The noradrenaline content was relatively constant in the different age groups, whereas the amount of both 5-hydroxytryptamine and dopamine showed considerable variations within the groups. The dopamine concentration was between 0.08 and 5.0 $\mu\text{g/g}$ in fetuses

and newborns, whereas it was extremely low in the adult animals. It appeared likely that noradrenaline was stored in adrenergic nerves, and dopamine in endocrine cells. The cellular localization of 5-hydroxytryptamine met with some difficulties because the yellow indole fluorescence, as observed microscopically, did not always correspond to the chemically determined content of 5-hydroxytryptamine. None the less, microspectrographic analysis of the specific fluorescence of the islet cells indicated that 5-hydroxytryptamine in some of these are stored together with dopamine and that 5-hydroxytryptamine fluorescence hereby could be masked by the light emitted from the dopamine fluorophore. These results represent the finding of a hitherto unknown localization of dopamine, namely in the endocrine pancreas, and emphasize the striking fact that two monoamines are stored in the same cell.

The great variations in dopamine content could later be explained. Cegrell (1967) found considerable amounts of dopamine in the pancreas of pigmented guinea-pigs, whereas the values in albinotic guinea-pigs were considerably lower. This correlation to pigmentation raises the question as to whether the dopamine is synthesized *de novo* in the pancreas, or is formed there from circulating dopa (cf. Cegrell 1967). The following facts support the view that dopamine is formed in the islet of Langerhans:

- 1) Dopamine seems to be a normal constituent of the islet cells in unpigmented pigs and exists there without noticeable variations in concentration (Cegrell et al. 1968).
- 2) The skin appears a conceivable source of dopa in pigmented guinea-pigs. In the pancreas a high dopamine concentration can

occur in nearly white guinea-pigs with only an isolated pigmented spot (Cegrell 1967).

3) Islet cells in many mammals, including the mouse, have an effective uptake mechanism for L-dopa, which in the cells is decarboxylated to dopamine (Cegrell 1968). In melanoma-carrying mice, whose tumours form large amounts of dopa, only few islet cells with specific catecholamine fluorescence can be demonstrated histochemically (unpublished results).

4) In experimental insulomas, dopa seems to be formed and metabolized into dopamine in the tumour (see papers X and XI). The finding of dopamine in endocrine pancreas is interesting also from another aspect. It is known that catecholamines can influence insulin release from the β -cells. This influence was earlier believed to be indirect and mediated by a mobilization and metabolism of glucose in other tissues. More recently it has been shown that adrenaline inhibits the insulin secretion by stimulating the α -receptors of the islet cells, whereas stimulation of the β -receptors produces the opposite effect. Dopamine has been proved to inhibit the insulin secretion from the β -cells in pancreas in vitro. It has also been demonstrated that adrenaline influences the metabolism of cyclic AMP in the islet of Langerhans; this nucleotide, in turn, influences the insulin secretion (for ref., see Mayhew et al. 1969). It would thus appear that catecholamines can influence the production of insulin, but the function in the pancreas of endogenous dopamine remains to discover.

Dopa and dopamine in insuloma

A transplantable, insulin forming insuloma in the golden hamster, described by Kirkman (1962), Grillo et al. (1967) and Sodoyez et al. (1967), has been investigated in paper VIII. In spectrophotofluorometric studies the tumour tissue was found to contain dopa, dopamine, and 5-hydroxytryptamine in amounts shown in Table 2, whereas noradrenaline and adrenaline could not be detected.

Table 2. The concentration of dopa, dopamine and 5-hydroxytryptamine in an islet-cell tumour of the golden hamster.

	µg/g	Number of determinations	Determination method
Dopa	0.19	4	Anton & Sayre 1964
Dopamine	2.6	4	Anton & Sayre 1964
Dopamine	2.8	4	Bertler et al. 1958
5-hydroxy-tryptamine	0.87	5	Bertler 1961

On spraying paper chromatograms of eluates from Dowex 50 with $K_3Fe(CN)_6$, only dopamine could be observed. If, on the other hand, the chromatograms were exposed to formaldehyde, two fluorescent spots appeared of which one on microspectrographic analysis had the same spectral characteristics as the fluorophore of dopamine. As to the other spot, the R_f -value and excitation/emission maxima at 400/430 nm gave no clue for identification. Apparently, it is not a catechol, because paper chromatography of eluates from Al_2O_3

showed only two spots, which fluoresced after formaldehyde treatment and whose Rf-values and spectra agreed with the fluorophores of dopa and dopamine, respectively. The fact that the unidentified compound could not be visualized with $K_3Fe(CN)_6$, indicates that the substance in question is not a catechol compound. Nor is it likely to be an indole derivative because fluorogenic indole derivatives have emission maxima at longer wave lengths than catechol derivatives (Björklund et al. 1972).

Naturally, some reservation is always called for when investigating tumour tissue in attempt to elucidate biochemical aspects in normal cells from which the tumour emanates. This is exemplified in the present study in that hamster insuloma cells display an intense formaldehyde induced fluorescence and contain dopa, dopamine and an unknown fluorogenic substance, whereas no monoamines seem to be present in the islet cells of adult hamster since they do not display any specific fluorescence (Cegrell 1968). Non the less, it is of great interest that dopamine and its immediate precursor dopa are found in hamster insuloma, as it supports the view that insulin forming cells can synthesize monoamine (cf. also papers X and XI).

The demonstration of 5-hydroxytryptamine in insuloma is also of interest because it has been shown (paper VII) that this amine, together with dopamine, is present in the islet of Langerhans in guinea-pigs.

Subcellular localization of dopamine in insuloma

Since cells containing biogenic amines also contain enzymes responsible for their catabolism and because they apparently exist in an active form, the amines must be kept functionally separated from the inactivating enzymes. It is known that catecholamines, like acetylcholine, is stored in special particles. The storage of catecholamines to certain cell components was first discovered in the suprarenal medulla from which Blaschko and Welch (1953), and Hillarp et al. (1953) isolated granula containing large amounts of catecholamines. These granula, which were isolated by gradient centrifugation, are smaller and have greater density than mitochondria. The storage mechanism does not appear to be specific for a particular amine. This is exemplified by the fact that suprarenal medulla granula, which normally store mainly noradrenaline and adrenaline, also store dopamine and 5-hydroxytryptamine on administering dopa and 5-hydroxytryptophan to rabbits (Bertler et al. 1960). In the following the subcellular localization of the monoamines in tumour cells was investigated. Differential centrifugation was carried out on tissue from hamster insuloma (paper IX). The particulate fractions and a small aliquote of the supernatant were analysed for their content of dopamine, 5-hydroxytryptamine, and histamine. Histamine was determined as described by Kurahashi and Fujiwara (1969). The percentage distribution of the amines thus obtained is presented in Table 3.

Table 3. Distribution in per cent of dopamine, 5-hydroxytryptamine, and histamine in sediments and supernatant after differential centrifugation of insuloma.

Amine	Sediment I (20,000 xg)	Sediment II (100,000 xg)	Supernatant	Number of determinations
Dopamine	16	15	69	9
5-hydroxy-tryptamine	52	25	23	3
Histamine	39 ; 32	49 ; 53	12 ; 15	2

The remainder of the supernatant was precipitated with ammonium sulphate. The precipitate was treated in two different ways: it was either dialyzed against a phosphate buffer (pH 7) or allowed to pass a column containing Sephadex G25. After dialysis 30 % of the dopamine in the supernatant remained in the ammonium sulphate precipitate, whereas a somewhat smaller amount of dopamine was found in the protein fraction after gel-chromatography. The caudate nucleus and the pancreas from pig and the suprarenal medulla from cow were used for comparison. In none of these tissues could dopamine be found in the precipitate after addition of ammonium sulphate. An unspecific adsorption of dopamine to protein did not occur in controls with dopamine added to serum albumin or blood plasma. Control tests were also carried out with liver capsule from cow, which contains a high concentration of dopamine localized to mast cells. These cells, which also occur in insuloma, are rich in heparin, known to form a complex with amines. In liver capsule tissue complex of this kind could not be precipitated with ammonium sulphate. In this

tumour, apparently, a considerable part of the dopamine is not bound to particles but relatively firmly associated with soluble protein, a phenomenon that has so far not been demonstrated in any other tissue.

The experiments further showed that in insuloma 5-hydroxytryptamine and histamine are particle bound to a much greater extent than is dopamine. The 5-hydroxytryptamine and histamine in the supernatant could not be precipitated with ammonium sulphate.

Metabolism of dopamine in insuloma

To see whether insuloma cells can synthesize and degrade dopamine, the enzyme activities involved must be investigated.

A. Tyrosine hydroxylase The biosynthesis of dopa from tyrosine was for a long time believed to be catalyzed by tyrosinase both in catecholamine and in melanin producing cells. However, the existence of another tyrosine hydroxylating enzyme has been demonstrated in the brain and the suprarenal gland of various mammals (Nagatsu et al. 1964). This enzyme, L-tyrosine hydroxylase, can probably also catalyze the hydroxylation of phenylalanine into tyrosine (Ikeda et al. 1967), but not the formation of melanin. In paper X it is demonstrated that insuloma tissue has a considerable tyrosine hydroxylating activity determined according to Nagatsu et al. (1964). In order to differentiate between the two enzymes, some inhibitors and activators were applied in the present work. The results are summarized in Table 4.

Table 4. The conversion of C-14-tyrosine to catechol derivatives in the islet cell tumour expressed as per cent of control in the presence of different substances.

Addition to the incubation medium		Number of determinations	% of control, mean value
Substance	Concentration (M)		
Tetrahydrofolic acid	10^{-4}	5	340
2,2'-Bipyridyl	10^{-3}	4	18
H 22/54	2×10^{-4}	4	40
Thiourea	10^{-4}	4	104
NSD 1015	10^{-4}	5	255

L-tyrosine hydroxylase for its activity requires oxygen, 2-amino-4--hydroxy-6,7-dimethyltetrahydropteridine (which can be replaced by tetrahydrofolic acid), and probably also Fe^{2+} . Bipyridyl and H22/54 (α -propylidopacetamide) inhibit L-tyrosine hydroxylase, but not tyrosinase. Thiourea, on the other hand, is a tyrosinase inhibitor. NSD 1015 (m-hydroxybenzylhydrazine) has been included because this hydrazine derivative is an effective inhibitor of dopa decarboxylase and accordingly promotes the accumulation of dopa in tissues in amounts sufficient for its identification (Cegrell et al. 1970). From Table 4 it is clear that the enzyme in question cannot be tyrosinase but that it satisfies the criteria for L-tyrosinehydroxylase.

B. Dopa decarboxylase The biosynthesis of dopamine from dopa is catalyzed by the enzyme dopa decarboxylase. In paper XI, the dopa decarboxylase activity was measured according to Bertler and

Rosengren (1959). A significant formation of dopamine occurred when dopa was added to tumour homogenates, a reaction which was strongly inhibited by NSD 1015. This shows that dopamine can be formed in the transplantable insuloma.

C. Monoamine oxidase (MAO) and catechol-O-methyltransferase (COMT) The incubation experiments in paper X revealed the presence of dihydroxyphenylacetic acid (DOPAC). Direct determinations of DOPAC according to Rosengren (1960) and homovanillic acid (HVA) according to Andén et al. (1963) in insuloma tissue showed that the tumour contained these two dopamine metabolites in concentrations in the range 0.2 - 0.6 µg/g. In this context it should be mentioned that the increase (mentioned under A above) of dopa on adding NSD 1015 to the incubates and the occurrence of HVA in the tumour indicates a rapid metabolism of dopa in the insuloma which is clearly shown by the finding (paper XI) that NSD 1015 given to tumour-carrying animals produced a rapid and pronounced increase in the dopa content of the tumour.

Paper XI verifies the occurrence of MAO-activity in the insuloma by determining the indole acetic acid formed from tryptamine. A significant MAO-activity was found, a result to be expected on the basis of the demonstration of DOPAC (paper X). Studies of COMT-activity have not as yet been carried out; the finding of HVA (paper X) is at present the only indication that this enzyme could occur in the insuloma.

Acknowledgements. This investigation has been supported by grants from The Swedish Medical Research Council (B72-14X-712-07B and B72-14X-56-08B) and from the Swedish Cancer Society.

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